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Interaction of GTP-binding proteins with calmodulin

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Two GTP-binding proteins (G_i and G_o), which were the substrates for islet-activating protein, pertussis toxin, were purified from bovine cerebral cortical membranes. Both G_i and G_o completely inhibited calmodulin-stimulated cyclic nucleotide phosphodiesterase activity. The same concentrations of these proteins, however, had no appreciable effect on the basal phosphodiesterase activity. The isolated $G_i\alpha$ and $\beta\gamma$ subunits of GTP-binding proteins were potent inhibitors of the calmodulin-stimulated phosphodiesterase activity, but $G_o\alpha$ was very weak. Therefore, the $\beta\gamma$ subunits were likely to be the major active molecules in the brain membranes. GTP-binding proteins were shown to bind directly to calmodulin in a Ca^{2+}-dependent manner by a gel permeation binding experiment.

GTP-binding protein *Calmodulin* *Cyclic nucleotide phosphodiesterase* *Protein interaction*

1. INTRODUCTION

Recent studies have demonstrated high concentrations of GTP-binding proteins in brain membranes, and two major proteins have been purified [1,2]. One is the inhibitory GTP-binding protein of adenylate cyclase (G_i), and the other is an analogous protein termed G_o by Sternweis and Robishaw [1]. Both α subunits bind GTP and are ADP-ribosylated by islet-activating protein, pertussis toxin. Both $\beta\gamma$ subunits from the two proteins seem to be identical [1]. These two proteins account for 1.5% of the membrane proteins [1], and G_o exists several-times more than G_i in brain membranes. Molecular mechanisms of the action of these GTP-binding proteins have not been clear except for the role of G_i in adenylate cyclase system [3].

Calmodulin is a Ca^{2+}-binding protein that modulates the action of numerous Ca^{2+}-dependent processes [4]. It is found in high concentrations in the central nervous system. Calmodulin exists in both cytoplasmic and particulate fractions of cells [5] and is associated with specific membranous components of neurons, including synaptic [6] and vesicular membranes [7]. Functionally, calmodulin has been shown to activate cyclic nucleotide phosphodiesterase [8], adenylate cyclase [9], and Ca^{2+},Mg^{2+}-ATPase [6] in brain. It has also been shown to be involved in Ca^{2+}-dependent phosphorylation of synaptic membranes [7], in axonal transport [10], and in the release of neurotransmitters [7].

In addition to calmodulin-regulated enzymes, various proteins and peptides including cytoskeletal proteins [4], opioid peptides [11], hormones [12], insect venoms [13] and seminalplasmin [14] have been shown to bind calmodulin or/and to inhibit the activity of calmodulin in a Ca^{2+}-dependent manner. But the biological significance of the binding of these proteins or peptides to calmodulin has not been clarified.

This study shows that GTP-binding proteins inhibit the calmodulin-stimulated phosphodiesterase activity and that this inhibition is apparently due to a direct Ca^{2+}-dependent interaction of GTP-binding proteins with calmodulin.

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2. MATERIALS AND METHODS

Calmodulin was purified from rat testis by the method of Yazawa et al. [15]. [^{3}H]Calmodulin was prepared by acetylation of rat testis calmodulin using [^{3}H]acetic anhydride (Amersham) [16].

Calmodulin-deficient phosphodiesterase was prepared from the soluble fraction of bovine cerebral cortex as described by Sharma and Wang [17]. Cyclic nucleotide phosphodiesterase activity was measured by the method described in [18].

G_i and G_o were purified from bovine cerebral cortex according to Sternweis and Robishaw [1]. Each subunit was resolved according to the method described in [19,20]. An equal amount of $\beta\gamma$ subunits was added to $G_i\alpha$ and $G_o\alpha$ preparations to form G_i and G_o, respectively. To determine GTP-binding protein, the binding of [^{35}S]guanosine 5′-(3-*O*-thio)triphosphate (GTPγS) (New England Nuclear) was measured essentially according to Northup et al. [21].

The binding of GTP-binding proteins to calmodulin was examined by the gel permeation binding technique of Hummel and Dreyer [22]. The preparation of G_i or G_o was subjected to Sephadex G-50 column (0.7 × 11 cm) equilibrated with [^{3}H]calmodulin (10000 cpm/ml) in 10 mM Hepes (pH 8.0), 0.5 mM dithiothreitol (DTT), 0.05% Lubrol PX, and 0.5 mM $CaCl_2$ or 0.5 mM EGTA, and the radioactivity of each fraction (105 μl) was counted.

3. RESULTS

Fig.1 shows the effects of GTP-binding proteins on cyclic nucleotide phosphodiesterase activity. Both G_i and G_o inhibited calmodulin-stimulated phosphodiesterase activity with IC_{50} values of 130 and 760 nM, respectively, but the same concentrations of these proteins had no significant effect on the basal phosphodiesterase activity. The effect of each subunit of G_i and G_o was also determined (fig.1). $G_i\alpha$ and $\beta\gamma$ subunits exerted potent inhibition on the calmodulin-stimulated activity but $G_o\alpha$ subunit was a very weak inhibitor. Therefore, the inhibitory effect of G_o seemed to be due to the effect of $\beta\gamma$ subunits. In the presence of lower concentrations of detergent, IC_{50} values of GTP-binding proteins on the enzyme activity were much less than those in the presence of 0.1% Lubrol PX.

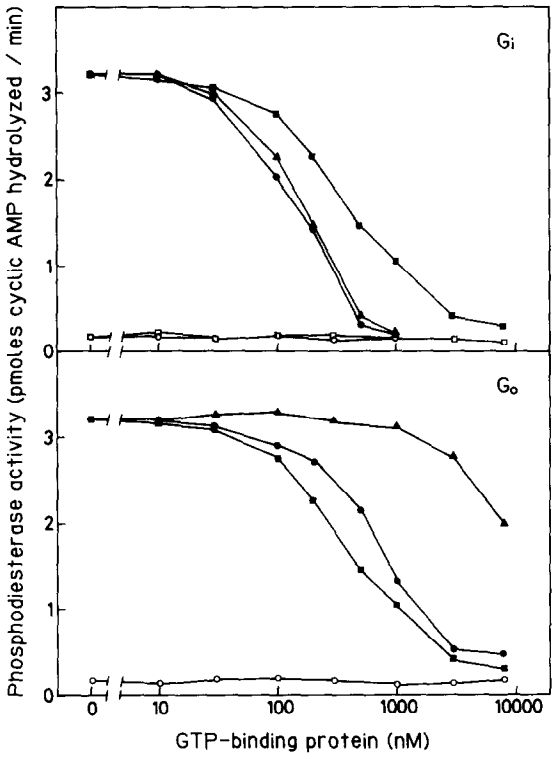

Fig.1. Effect of GTP-binding proteins and their subunits on phosphodiesterase. Phosphodiesterase activity was measured in the reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 2 μM cyclic [^{3}H]AMP, 0.1% Lubrol PX, 10 nM calmodulin, 0.5 mM $CaCl_2$ (●, ▲, ■) or 0.5 mM EGTA (○, □), and varying concentrations of GTP-binding proteins, G_i or G_o (●,○), α subunit (▲), and $\beta\gamma$ subunits (■, □). The reaction was started by the addition of the calmodulin-deficient phosphodiesterase preparation (0.22 μg protein), and was carried out for 10 min at 30°C.

For example, IC_{50} values of G_i and G_o were 35 and 65 nM, respectively, at a Lubrol concentration of 0.0025%.

The inhibitory effects of G_o on calmodulin-stimulated phosphodiesterase activity could be overcome by increasing concentrations of calmodulin (fig.2); higher concentration of calmodulin was required to antagonize the inhibitory effects of 4 μM G_o than of 1 μM G_o. Similar results were observed with G_i (not shown).

To determine whether GTP-binding proteins bind directly to calmodulin, the gel permeation binding experiment of Hummel and Dreyer [22]

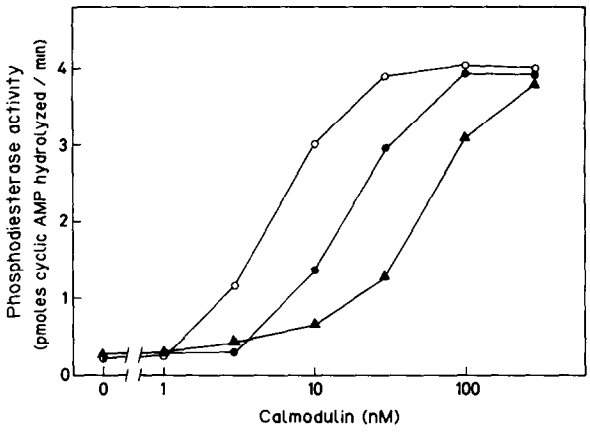

Fig.2. The reversal of the inhibitory effect of G_o on calmodulin-stimulated phosphodiesterase by increasing concentrations of calmodulin. Phosphodiesterase activity was measured in the reaction mixture containing 0.5 mM $CaCl_2$ and varying concentrations of calmodulin in the presence (●, 1 μM; ▲, 4 μM) or absence (○) of G_o.

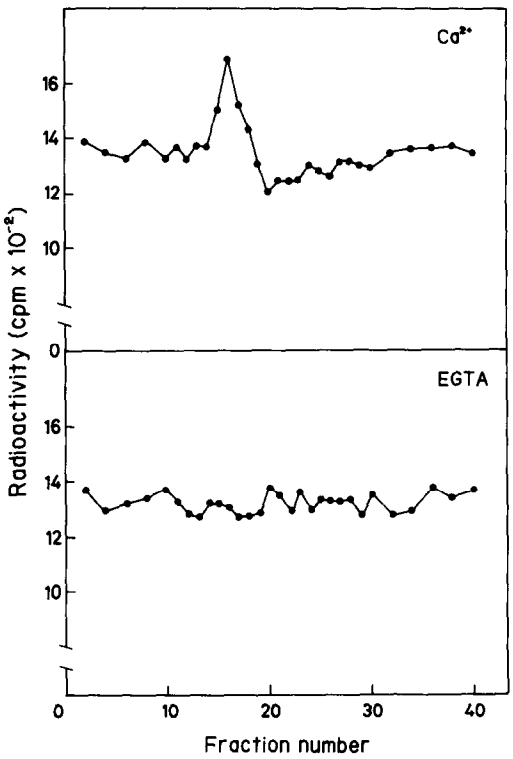

Fig.3. Elution profiles of G_o on Sephadex G-50 column equilibrated with the buffer containing [^{3}H]calmodulin. Sephadex G-50 column (0.7 × 11 cm) was equilibrated with the buffer containing 10 mM Hepes (pH 8.0), 0.5 mM DTT, 0.05% Lubrol PX, [^{3}H]calmodulin and 0.5 mM $CaCl_2$ or 0.5 mM EGTA at 25°C. G_o (2 nmol) was applied to the column and each fraction was analyzed for radioactivity.

was carried out with Sephadex G-50 column equilibrated with [^{3}H]calmodulin. Fig.3 illustrates a typical chromatogram obtained in a Ca^{2+}-containing buffer following the application of G_o. Fig.3 also shows that the binding of G_o to calmodulin appears Ca^{2+}-dependent, since EGTA prevents the formation of the G_o-calmodulin complex. Similar results were observed with G_i (not shown). These results indicate that GTP-binding proteins bind directly to calmodulin in a Ca^{2+}-dependent manner.

4. DISCUSSION

Both G_i and G_o prevented the activation of cyclic nucleotide phosphodiesterase by calmodulin. The results from the gel permeation binding experiments of Hummel and Dreyer [22] indicated that GTP-binding proteins bound to calmodulin in a Ca^{2+}-dependent manner. G_o was a less potent inhibitor of phosphodiesterase than G_i, but it exists at much higher concentration in the brain membranes. Because the inhibitory effect of G_o was derived from $\beta\gamma$ subunits, $\beta\gamma$ subunits should be the major molecules to interact with calmodulin. The affinities of the GTP-binding proteins for calmodulin are not so high as that of calmodulin-regulated enzyme or various cytoskeletal calmodulin-binding proteins [4]. But the IC_{50} values of G_i and G_o were lower than those of opioid peptides [11], and were the same order with insect venom peptides [13] and seminalplasmin [14] which were recently reported to interact with calmodulin.

All assays of phosphodiesterase in this study were carried out in the presence of 0.1% Lubrol PX. When the assay was performed in the presence of lower concentrations of detergent, IC_{50} values of G_i and G_o were less than those in the presence of 0.1% Lubrol. These phenomena suggest a hydrophobic interaction between calmodulin and GTP-binding proteins. Furthermore, the $\beta\gamma$ subunits seem to be hydrophobic proteins because they aggregate in the absence of detergent, while $G_i\alpha$ and $G_o\alpha$ subunits remain monomeric in the

same conditions [23]. Therefore, the inhibition of calmodulin-stimulated phosphodiesterase by $\beta\gamma$ is probably due to the hydrophobicity of this dimer like the inhibition obtained with neuroleptics [24].

G_i and G_o account for 1.5% of the membrane proteins [1], viz. 15 μg/mg of membrane proteins. On the other hand, the concentration of calmodulin in the particulate fractions of rat cerebral cortex was reported to be 2.7 μg/mg of membrane proteins [5]. This is a similar concentration to that of GTP-binding proteins.

The physiological significance of the interaction of calmodulin with GTP-binding proteins is still unclear. However, it may suggest a novel function of calmodulin and GTP-binding proteins because both proteins exist at high concentration in the brain membranes. It presumably includes complex regulatory system related to Ca^{2+} mobility in the cell membranes.

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